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EPIDERMAL GROWTH FACTOR STIMULATES TYROSINE PHOSPHORYLATION OF HUMAN GLUCOCORTICOID RECEPTOR IN CULTURED CELLS

Kanury V.S. Rao and C. Fred Fox*

Department of Microbiology and Molecular Biology Institute,
University of California at Los Angeles, Los Angeles, CA 90024

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Human breast epithelial HBL100 cells, which bind both epidermal growth factor (EGF) and glucocorticoids, were labelled to steady state specific activity with ^{32}Pi and the glucocorticoid receptor was immunoprecipitated from cell lysates with polyclonal antiserum GR884. Immuneprecipitated receptor was resolved by $NaDodSO_4$-polyacrylamide gel electrophoresis and identified by autoradiography. Immunoprecipitated receptor also was characterized by western blot analysis and affinity labelling with [^3H]dexamethasone-21- mesylate. Phosphoamino acid analysis of ^{32}P-glucocorticoid receptor revealed 89% phosphoserine and 11% phosphotyrosine. Treatment of steady state ^{32}Pi-labelled cells with EGF stimulated total and alkali-stable phosphorylation in the 97 kDa receptor band by about 35%. Prior incubation with dexamethasone inhibited EGF stimulated, alkali-stable phosphorylation of the 97 kDa glucocorticoid receptor band.

The progesterone, estrogen and glucocorticoid receptors are phosphoproteins(1-4). Direct demonstration of glucocorticoid receptor phosphorylation has been obtained following ^{32}Pi-labelling of intact mouse fibroblast L-cells (3) or injection of adrenalectomized rats with ^{32}Pi (5). Phosphoamino acid analysis revealed only phosphoserine residues in the former case (3).

Glucocorticoid receptor phosphorylation can modify its ligand binding activity. The glucocorticoid binding capacity of soluble preparations from mouse fibroblast L-cells was inactivated by purified calf intestinal alkaline phosphatase, and this inactivation was blocked by phosphatase inhibitors such as molybdate and fluoride (6). Inactivation of unoccupied receptor in the soluble fraction of L-cell homogenates is catalyzed to a greater extent by highly purified rabbit muscle protein phosphatase than by purified calf intestinal alkaline phosphatase (7). Rabbit muscle protein phosphatase has prefered activity towards phosphoserine residues, and alkaline phosphatase has preference for phosphotyrosine residues (8), suggesting a correlation between

*To whom correspondence should be addressed.

Abbreviations Used: EGF, epidermal growth factor; dexamethasone, 9α-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione; Pi, inorganic phosphate; $NaDodSO_4$, sodium dodecyl sulfate.

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phosphorylation on serine residues and maintenance of glucocorticoid binding activity by the mouse L-cell receptor (3). These and other properties of the system have led to the hypothesis that phosphorylation of the glucocorticoid receptor helps maintain it in a conformation favoring a reduced state necessary for ligand binding activity (7,9).

In a preliminary report we described evidence for an EGF-induced decrease in progestin binding of cultured human breast carcinoma cells (10). In recent experiments we found that EGF also decreased glucocorticoid binding in human breast-derived HBL100 cells (Rao and Fox, manuscript in preparation). It was therefore of interest to test for effects of EGF on glucocortiocid receptor phosphorylation. This report describes results with HBL100 cells, an epithelial, non-tumorigenic line derived from human milk (11).

MATERIALS AND METHODS

Materials: [1,2,4,6,7-^{3}H]Dexamethasone (70 Ci/mmol) was purchased from Amersham Corporation (Arlington Heights, IL), and [6,7-^{3}H(N)]dexamethasone-21-mesylate (49 Ci/mmol), from New England Nuclear (Boston, MA). Unlabelled dexamethasone, leupeptin, dithiothreitol, EDTA, EGTA and PMSF were purchased from Sigma Chemical Co. (St. Louis. MO.). [^{32}P]Orthophosphate (285 Ci/mg P) was obtained from ICN Radiochemicals (Irvine, CA.). Unless stated otherwise, all other chemicals were reagent grade.

Rabbit polyclonal antiserum, GR884 (12,13), was a gift from R.M. Evans (Salk Inst., San Diego, CA).

Cells and Cell culture: HBL100 cells (obtained from W.L.McGuire, Univ. of Texas Health Science Centre, San Antonio, Texas) were maintained in 55 cm^2 polystyrene petri plates in McCoy'c 5A (modified) culture medium (GIBCO) supplemented with L-glutamine (2 mM), 10% fetal bovine serum (GIBCO, Chagrin Falls, OH) and 10 μg/ml gentamycin at 37^o in a humidified atmosphere of 5% CO_2 and 95% air. The cells, plated at approximately 10^4 cells/cm^2, were grown to near confluency and passed once weekly, with a change in the culture medium on the third day after passage. For adherent cell binding studies, cells were grown in 24-well polystyrene dishes (Corning Glass Works, Corning, N.Y.) for four days to $0.7x10^5$ cells/cm^2 (60% confluency).

[^{32}P]Orthophosphate labelling: HBL100 cells were plated in six well, 9.6 cm^2 dishes (COSTAR, Cambridge, MA) at 10^5 cells/well and grown for three days until 60% confluency. The culture medium was removed and replaced with 2 ml/well of medium 199 (phosphate free), supplemented with L-glutamine and containing 10% fetal bovine serum (dialyzed extensively against phosphate free medium 199) and 10 μg/ml gentamycin. After the cells had been incubated for 24 hr at 37^o, [^{32}P]orthophosphate was added at 0.5 mCi/ml of culture medium. After an additional 4h. at 37^o, another 0.5 mCi/ml of ^{32}Pi was added and the cells were incubated for 18 hr.

Immunoprecipitation of the glucocorticoid receptor: [^{32}P]Orthophosphate labelled cells were chilled to 0^o and lysed in 1 ml modified RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% Deoxycholate, 0.1% $NaDodSO_4$ and 80 μg/ml leupeptin, pH 7.2 containing 10 mM sodium molybdate and 1mM sodium vanadate as phosphatase inhibitors). After 5 min the lysate was clarified in an Eppendorf centrifuge (15,600g x 10 min.) and the resulting supernatant fraction was removed and incubated for 30 min at 22^o with a preformed complex (60 min, 22^0) of rabbit IgG (20 μl, 1 μg/ml) with formalin fixed Staphylococcus aureus (50 μl of a 10% w/v suspension in phosphate buffered saline) (14). The suspension was centrifuged (15,600gx5min) and the resultant supernatant fraction was incubated with a preformed complex of GR884 antiserum (5 μl, 540 μg/ml) and Staphylococcus aureus (50 μl) at 4^0. After 4 hr, the S. aureus cells were sedimented and the pellet washed three times with 1ml of RIPA buffer. Bound proteins were solubilized by boiling for 10 min in 100 μl of sample buffer [50 mM Tris, 3% sodium dodecyl sulfate, 20% glycerol (v/v) and 5% 2-mercapto ethanol (v/v)].

Phosphoproteins were resolved by electrophoresis on a 7.5 % polyacrylamide gel and identified by autoradiography. To selectively reveal tyrosine phosphorylated bands, gels were treated with 1M KOH for 2hr at 55^0 (15) and subsequently neutralized (1M HCl, 15 min at 22^0) prior to drying.

Phosphoamino acid analysis: The radiolabelled glucocorticoid receptor band was excised from the gel, eluted and hydrolyzed in 6M HCl at 108^0 for 2hr (16). After addition of phosphoserine, phosphothreonine and phosphotyrosine reference standards, the hydrolysate was resolved in two dimensions by electrophoresis at pH 1.9 (2.5h, 400V) and at pH 3.5 (2h, 400V) on 20 cm x 20 cm thin layer (0.1 mm) cellulose plates and analyzed for phosphorylated amino acids (17). Reference standards were visualized by staining with ninhydrin.

RESULTS

[^{3}H]Dexamethasone binding to HBL100 cells revealed a single class of high affinity binding sites (Kd = 2 nM) with $1.5 \pm 0.2 \times 10^5$ sites per cell (data not shown). Equilibrium binding of ^{125}I-EGF (30 nM, 4^0, 4 hr) revealed $4.1 \pm 0.4 \times 10^4$ EGF-binding sites per cell, in agreement with published values (18).

^{32}Pi-labelled HBL100 cells were incubated in the presence or absence of 3 nM EGF for 1 hr at 37^0. The cells were lysed with modified RIPA buffer and glucocorticoid receptor was immunoprecipitated with polyclonal antiserum GR884. This well characterized antiserum was raised against the human lymphoid receptor (12) and has been used to identify cDNA clones expressing human glucocorticoid receptor (13). Immunoprecipitation from cell lysates with this antiserum, followed by $NaDodSO_4$-polyacrylamide gel electrophoresis and subsequent alkali-treatment of the gel to visualize phosphotyrosine content (15), revealed a major ^{32}P-labelled band at 97 ± 1 kDa upon autoradiography (see Fig. 2 as an example); this agrees with values reported for the glucocorticoid receptor by others (13,19). Autoradiography of gels that were not treated with alkali also revealed the 97 kDa band as the major ^{32}P-labelled band (data not shown). An additional ^{32}P-band was present at 130 kDa, as were other lower molecular weight bands revealed on alkali treated gels (Fig. 2). Further characterization of the 97 kDa band as that with mobility of the glucocorticoid receptor was obtained from Western blot analysis (20) (MW = 97.3 kDa, data not shown) and by immunoprecipation of [^{3}H]dexamethasone-21-mesylate affinity labelled receptor from the soluble fraction of HBL100 cell homogenates (MW = 97 kDa, Fig. 1).

EGF treatment stimulated alkali stable phosphorylaton of the 97 kDa band (Table 1). The average net increase in phosphorylation caused by EGF was $39 \pm 4\%$ and was significant as determined by the Student T-test ($p < 0.003$). A comparable stimulation by EGF was observed in total phosphorylation of the 97 kDa band which was determined from gels that were not alkali treated (the average increase was by 1.35 ± 0.15 fold ; $p < 0.02$ where $n = 3$). Longer incubation

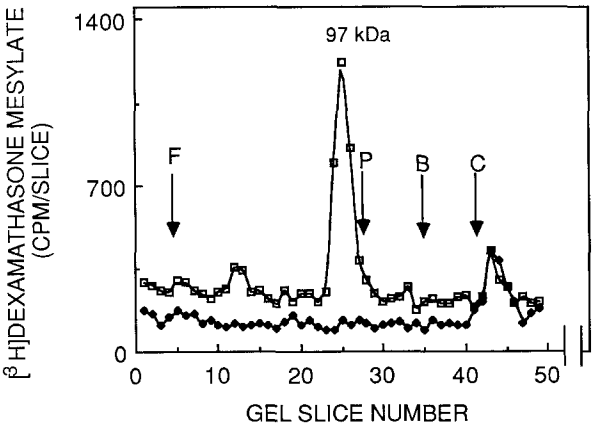

Fig. 1. Immunoprecipitation of [^{3}H]dexamathasone-21-mesylate affinity labelled glucocorticoid receptor from the cytosol of HBL100 cells. HBL100 cells grown in glass roller bottles were suspended and homogenized (Dounce, 50 strokes) in one volume of homogenizing buffer (10mM Tris-HCl, 1mM Na_2EDTA, 5mM EGTA, 2mM PMSF, 10% glycerol, 10mM sodium molybdate, 1mM sodium vanadate, 10mM ATP and 40μg/ml leupeptin). The cell homogenate was centrifuged for 40,000 g.min. and the supernatant clarified further by a $6x10^6$ g.min. centrifugation. Aliquots of 500 μl were incubated for 4 hr at 0^0 with 200 nM [^{3}H]dexamathasone-21-mesylate either in the presence (■) or absence (□) of 20 μM unlabelled dexamathasone. Reactions were quenched by addition of 2mM dithiothreitol. Glucocorticoid receptor was then immunoprecipitated and analyzed by gel electrophoresis as described in Materials and Methods. Lanes containing samples were cut into 2mm slices, solubilized in a mixture of 0.4 ml 30% H_2O_2 and 0.2 ml $HClO_4$ (60^o, overnight) and the radioactivity measured. F, ferritin (220,000 Da.); P, phosphorylase b (94,000 Da); B, bovine serum albumin (67,000 Da.); C, catalase (60,000 Da.).

times with EGF (up to 6 hr) did not induce additional phosphorylation of the 97 kDa band, but concentrations of dexamethasone sufficient to saturate the glucocorticoid receptor inhibited EGF stimulated alkali-stable phosphorylation of the 97 kDa band (Fig. 2, lanes c to f). We have not

Table 1. Effect of EGF on the alkali-stable phosphorylation of glucocorticoid receptor

EXPT.	ADDITIONS	^{32}Pi in 97 kDa band (cpm x 10^{-2})
1.	NONE	6.4
	3 nM EGF	8.7
2.	NONE	9.0
	1 nM EGF	12.9
	3 nM EGF	12.5

Cells labelled to the steady state with ^{32}Pi-labelled were incubated with or without EGF for 60 min at 37^0 and lysed with 1 ml of modified RIPA buffer. The glucocorticoid receptor was immunoprecipitated with antiserum GR884 and resolved by $NaDodSO_4$-polyacrylamide gel electrophoresis. The gel was alkali treated and the position of the 97 kDa band determined by autoradiography. Radioactivity of the glucocorticoid receptor band was determined by Cerenkov counting of excised slices. See Materials and Methods for details.

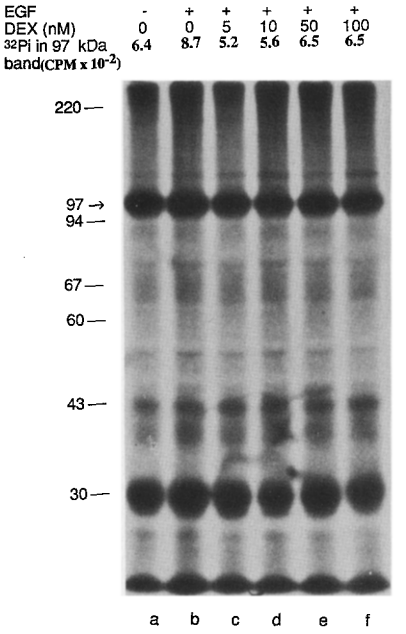

Fig. 2. Effect of EGF on alkali-stable phosphorylation of the glucocorticoid receptor in the presence or absence of dexamethasone. HBL100 cells were radiolabelled with ^{32}Pi (see materials and methods). Dexamethasone was added to achieve the concentrations shown and the cells were incubated at 22^0 for 15 min. After 3 nM EGF was added, the cells were incubated for an additional 60 min at 37^0. The cells were chilled and lysed, and the glucocorticoid receptor was immuneprecipitated and analyzed by $NaDodSO_4$-polyacrylamide gel electrophoresis. The gel was stained, destained ,alkali-treated and dried as described in Materials and Methods. The dried gel was exposed to X-ray film for 18 hr.

determined the effect of glucocorticoid receptor occupancy on EGF-induced phosphorylation of serine residues in the 97 kDa glucocorticoid receptor band.

Since published results have described only serine phosphorylation of the glucocorticoid receptor (3), phosphoamino acid analysis was performed to determine if the alkali stable radioactivity present in the 97 kDa glucocorticoid receptor band was phosphotyrosine. HBL100 cells were labelled to steady state specific activity with ^{32}Pi and incubated in the presence or absence of 3 nM EGF at 37^0 for 1 hr. The cells were lysed with modified RIPA buffer and treated with polyclonal antiserum GR884. Proteins in the resulting immune-complex were resolved by $NaDodSO_4$-polyacrylamide gel electrophoresis and the 97 kDa glucocorticoid receptor band was excised from the gel, extracted, and hydrolyzed with acid. The resulting digest was analysed for ^{32}P-labelled amino acids, and the results from a typical experiment are shown in Fig. 3. While the major radiolabelled phosphoamino acid is phosphoserine, there was a significant contribution from phosphotyrosine in both EGF treated and untreated cells (Fig. 3, Table 2). Treatment with EGF resulted in increased phosphorylation on both serine and tyrosine residues (Fig. 3); as a

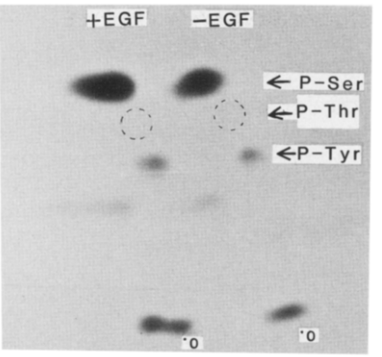

Fig. 3. Analysis of phosphorylated amino acids in glucocorticoid receptor. The glucocorticoid receptor band from EGF (3nM) treated or untreated cells was excised from the gel (see legend to Fig.2.), and the 97 kDa protein band was eluted and hydrolyzed with 6M HCl at 108° for 2h. Phosphorylated amino acids were separated in two dimensions as described in Materials and Methods. Radioactive regions were identified by autoradiography and analyzed by Cerenkov counting (Table 2.). P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine. The additional radioactive regions correspond to undigested peptides.

consequence, there was relatively little enhancement in phosphotyrosine content relative to that of phosphoserine (Table 2). The average increase in serine and tyrosine phosphorylation resulting from EGF treatment averaged 30% in phosphoserine and 40% in phosphotyrosine in three independent experiments.

DISCUSSION

Immunoprecipitation from lysates of steady state ^{32}Pi-labelled HBL100 cells with polyclonal antiserum GR884 (12,13) gives, on NaDodSO$_4$-polyacrylamide gel electrophoresis and autoradiography, a major ^{32}P-protein band at 97 kDa, the mobility of the glucocorticoid receptor (13,19). Phosphoamino acid analysis of the 97 kDa ^{32}P-protein band revealed a composition of 89% phosphoserine residues and 11% phosphotyrosine residues (Table 2). This, to our knowledge, is the first report describing the presence of phosphotyrosine residues associated with

Table 2. Relative abundance of phosphoamino acids of glucocorticoid receptor from EGF treated or untreated cells

EGF (3 nM)	p-Serine§ (%)	p-Threonine (%)	p-Tyrosine§ (%)
-	88.7 ± 1.5	ND*	11.3 ± 1.0
+	87.8 ± 1.4	ND*	12.3 ± 1.7

* ND, none detected.
§ Mean values (± standard deviation) of three independent experiments are presented; see Fig. 3 for experimental details.

glucocorticoid receptor. In their studies on mouse L-cell derived glucocorticoid receptor, Housley and Pratt detected only phosphoserine residues following phosphoamino acid analysis of affinity purified ^{32}P-labelled receptor (3). The difference between their results and ours does not reside in conditions for acid hydrolysis. It could lie in the cells used or in methods of receptor preparation since they did not describe the inclusion of phosphatase inhibitors during homogenization in the L-cell study (3).

EGF treatment resulted in enhanced phosphorylation of protein in the 97 kDa band (Table 1) on both serine and tyrosine residues (Fig. 3). Increased tyrosine phosphorylation could be due to a direct action of EGF receptor kinase. Enhanced serine phosphorylation on proteins has ocurred during EGF treatment in other cellular studies (21- 25) and is a result of action of other cellular kinase(s) stimulated by EGF binding.

In recent experiments we have observed a 35% decrease in dexamethasone binding within minutes of EGF treatment of HBL100 cells (Rao and Fox, manuscript in preparation). Furthermore, EGF addition did not lead to dissociation of [^{3}H]dexamethasone already associated with the glucocorticoid receptor. In this report we show that alkali-stable phosphorylation of the 97 kDa glucocorticoid receptor band was not stimulated by EGF in cells where glucocorticoid receptor was occupied with dexamethasone (Fig. 2). This evidence supports a direct causal relationship between enhanced 97 kDa protein phosphorylation on tyrosine residues and decreased dexamethasone binding occurring in response to EGF treatment of HBL100 cells.

Pratt and coworkers have proposed that maintenance of glucocorticoid receptors in a phosphorylated state is obligatory for glucocorticoid binding (7,9). This is supported by observations that the ligand binding capacity of soluble glucocorticoid receptor preparations from L-cells can be inactivated by treatment with highly purified alkaline phosphatase, that this inactivation is prevented by phosphatase inhibitors (7), and that the glucocorticoid binding capacity of the soluble fraction from rat thymocytes could also be stabilized by ATP addition (9). It will be of interest to determine if serine and tyrosine phosphorylations have opposing effects on the binding activity of the glucocorticoid receptor.

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